5430–5438 *Nucleic Acids Research, 2007, Vol. 35, No. 16* *Published online 15 August 2007*
doi:10.1093/nar/gkm576

Repeat-associated siRNAs cause chromatin silencing of retrotransposons in the *Drosophila melanogaster* germline

Mikhail S. Klenov[1], Sergey A. Lavrov[1], Anastasia D. Stolyarenko[1], Sergey S. Ryazansky[1], Alexei A. Aravin[2], Thomas Tuschl[2] and Vladimir A. Gvozdev[1,*]

[1]Department of Molecular Genetics of Cell, Institute of Molecular Genetics, Russian Academy of Sciences, Moscow 123182, Russia and [2]Howard Hughes Medical Institute, Laboratory of RNA Molecular Biology, the Rockefeller University, 1230 York Avenue, Box 186, New York, New York 10021, USA

Received May 19, 2007; Revised July 15, 2007; Accepted July 15, 2007

ABSTRACT

Silencing of genomic repeats, including transposable elements, in *Drosophila melanogaster* is mediated by repeat-associated short interfering RNAs (rasiRNAs) interacting with proteins of the Piwi subfamily. rasiRNA-based silencing is thought to be mechanistically distinct from both the RNA interference and microRNA pathways. We show that the amount of rasiRNAs of a wide range of retroelements is drastically reduced in ovaries and testes of flies carrying a mutation in the *spn-E* gene. To address the mechanism of rasiRNA-dependent silencing of retrotransposons, we monitored their chromatin state in ovaries and somatic tissues. This revealed that the *spn-E* mutation causes chromatin opening of retroelements in ovaries, resulting in an increase in histone H3 K4 dimethylation and a decrease in histone H3 K9 di/trimethylation. The strongest chromatin changes have been detected for telomeric *HeT-A* elements that correlates with the most dramatic increase of their transcript level, compared to other mobile elements. The *spn-E* mutation also causes depletion of HP1 content in the chromatin of transposable elements, especially along *HeT-A* arrays. We also show that mutations in the genes controlling the rasiRNA pathway cause no derepression of the same retrotransposons in somatic tissues. Our results provide evidence that germinal Piwi-associated short RNAs induce chromatin modifications of their targets.

INTRODUCTION

A high level of transposable element expression is usually deleterious for the organism, leading to mutations and chromosomal rearrangements. Therefore, activity of mobile elements is thought to be under keen cellular control. Silencing of *Drosophila* selfish elements is realized through the short RNA species, called repeat associated short interfering RNAs (rasiRNAs) (1–5) and also Piwi-interacting RNAs (piRNAs) (6). piRNAs play evolutionarily conserved roles in the regulation of transposable elements in insects, mammals and zebrafish (7–9) and are accumulated specifically in the germline (9–12).

In *Drosophila*, the rasiRNA pathway requires members of the 'Piwi subfamily' of Argonaute proteins Piwi, Aubergine (Aub) and Ago-3 (2–6) but not the 'Argonaute subfamily' members, Ago1 or Ago2 (3), which guide microRNA and siRNA functions, respectively (13). rasiRNAs of 24–28 nt in length are longer than 21–22 nt siRNAs derived from dsRNA or 21–23 nt endogenous microRNAs (1,2). The increased length of rasiRNAs has aroused a suggestion of a peculiar mechanism of their formation (2). In flies neither Dicer-1, which makes microRNAs, nor Dicer-2, which produces siRNAs, are implicated in rasiRNA formation (3). Recent publications support a model in which discrete heterochromatic loci produce rasiRNAs that are predominantly antisense to transposons. The antisense rasiRNAs are bound by Piwi and Aub proteins and guide formation of sense rasiRNAs by cleavage of sense transposon transcripts (5,6).

It was demonstrated that short interfering RNAs are implicated in chromatin modifications, such as methylation of histone H3 K9, in yeast, plants and animal somatic cells (14–17). However, it remains unknown

*To whom correspondence should be addressed. Tel: 09 51 96 00 12; Fax: 09 51 96 02 21; Email: gvozdev@img.ras.ru
Present address:
Alexei A. Aravin, Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York, 11724, USA.

whether chromatin-based silencing of selfish elements may be realized in the germline by Piwi-interacting RNAs, in particular by rasiRNAs in flies.

We show that the significantly reduced abundance of rasiRNAs derived from a wide range of transposable elements in *spn-E* mutant ovaries is accompanied by the increase of H3 K4 dimethylation, decrease of H3 K9 di/trimethylation and depletion of HP1 content in the chromatin of retrotransposons. We demonstrate that rasiRNA-mediated silencing of tested retrotransposons takes place in ovaries, where it is necessary to protect the genome against transposon-induced mutations in progeny, but not in somatic tissues.

MATERIALS AND METHODS

Drosophila strains

Strains bearing *spn-E*^{1}, *piwi*^{2} and *armi*^{1} mutations were *ru*^{1} *st*^{1} *spn-E*^{1} *e*^{1} *ca*^{1}/TM3, *Sb*^{1} *e*^{s} (point mutation in helicase domain of Spn-E), *ru*^{1} *st*^{1} *spn-E*^{hls3987} *e*^{1} *ca*^{1}/TM3, *Sb*^{1} *e*^{s} (P-element insertion into *spn-E*) (18,19), *piwi*^{2}/*CyO* (P-*ry11* transposon insertion) (20) and *armi*^{1}/TM3 (P-element insertion) (21), respectively. P-element transformed flies carrying the *copia*LTR-*lacZ* construct were kindly provided by E. G. Pasyukova. Discrimination in X-gal staining experiment of homo- and heterozygous larvae carrying *spn-E*^{1}, *piwi*^{2} and *armi*^{1} mutations was done using GFP-expressing balancers CyO, P{w^{+m} = hsp70: GAL4}P{w^{+m} = UAS: GFP} and TM3, *Sb*^{1} *e*^{s} P{w^{+m} = hsp70: GAL4}P{w^{+m} = UAS: GFP}.

RT–PCR analysis

Total RNA was isolated from dissected ovaries or carcasses using Trizol reagent (Gibco BRL). The first strand of cDNA was synthesized using SuperScript II reverse transcriptase (Gibco BRL) and oligo(dT) primer or specific primer according to the manufacturer's instructions. cDNAs were analyzed by real-time quantitative PCR using SYBR Green. For PCR the following primers were used: 5′-CCGTGGTCAACTTCACCAG CTC-3′ (adh d2) and 5′-TCCAACCAGGAGTTGA ACTTGTGC-3′ (adh r2), corresponding to GenBank sequence AE003410.1 for *Adh* gene; 5′-TCCGCCCAG CATACAGGC-3′ (rp49 s2) and 5′-CAATCCTCGTTGG CACTCACC-3′ (rp49 as2), corresponding to GenBank sequence Y13939 for *rp49* gene; 5′-GCATGAGAGG TTTGGCCATATAAGC-3′ (cop-s) and 5′-GGCCCACA GACATCTGAGTGTACTACA-3′ (cop-as), corresponding to GenBank sequence XO4456 for *copia*; 5′-CGCAA AGACATCTGGAGGACTACC-3′ (Het-s2) and 5′-TGC CGACCTGCTTGGTATTG-3′ (Het-as2), corresponding to GenBank sequence U06920 for *HeT-A*; 5′-TGAAA TACGGCATACTGCCCCCA-3′ (I el s2) and 5′-GCTG ATAGGGAGTCGGAGCAGATA-3′ (I el as2), corresponding to GenBank sequence M14954 for *I* element.

X-gal staining and β-gal activity assay

X-gal staining and β-gal activity assays were performed according to protocols described previously (2,22). Samples containing 5–15 pairs of ovaries dissected from 1 to 3-days-old females or 4–15 carcasses were used for β-gal activity assay. Measurements of β-gal activity were normalized to the total protein evaluated by the Bio-Rad protein assay kit.

Short RNA cloning and annotation

RNA preparation was performed as previously described (23). Total RNA was isolated from adult ovaries and testes. Cloning of miRNAs was performed as described (24). Characterization of cloned small RNAs was performed using local NCBI-BLAST 2.2.13 (25) against the canonical sequences of transposable elements (http://www.fruitfly.org/p_disrupt/datasets/ASHBURNER/D_mel_transposon_sequence_set.fasta); *Su(Ste)* repeats (GenBank accession no. X59157|H-, Z11734|H- and Z11735|H-); miRNAs (http://microrna.sanger.ac.uk/sequences/, Release 8.0), tRNA (http://lowelab.ucsc.edu/GtRNAdb/Dmela/) and rRNA (GenBank accession no. M21017). Only hits with 95% and higher similarity to transposable elements and *Su*(*Ste*) sequences and 100% similarity to other sequences were used. Parsing of results was done using corresponding BioPerl modules (26).

Chromatin IP assay

Ovaries were dissected from 1 to 10-days-old females in 1X PBS and stored in 1.5 ml tube on ice during isolation (up to 2 h). PBS solution was removed after centrifugation (3500 r.p.m. 1–2 min). 10 mg of material (about 150 ovaries or 100 carcasses) was used for one IP reaction. The chromatin IP assay was performed as described previously (27), using polyclonal rabbit antibodies (Upstate): Anti-dimethyl-Histone H3 Lys4 (#07-030), Anti-dimethyl-Histone H3 Lys9 (#07-441), Anti-trimethyl-Histone H3 Lys9 (#07-523) and anti-HP1 (PRB-291C Covance innovative). Anti-TAF1 was kindly provided by G. Cavalli. DNA precipitates were amplified by semiquantitative PCR in the presence of αP^{32} dATP or real-time quantitative PCR. PCR product quantities were normalized to input and relations to a fragment of intergenic spacer in the 60D region were calculated. No identified or predicted genes are located 2.5 kb upstream and 4.3 kb downstream of the 60D amplified fragment. The TRANSFAC database search found no binding sites for any known chromatin proteins and transcriptional factors in the fragment. Final enrichment values of sample PCR products were calculated using the following expression: $E(\text{product})^{\text{sample}}$ $^{*}E(60D)^{\text{input}}/E(60D)^{\text{sample}}$ $^{*}E(\text{product})^{\text{input}}$. The following primers were used for PCR analysis in ChIP: 5′-CAACA CTACTTTATATTTGATATGAATGGCC-3′ and 5′-CGAAAGGGGGATGTGCTGC-3′ for amplification of the promoter region of *copia*LTR-*lacZ* construct; 5′-CAACACTACTTTATATTTGATATGAATGGCC-3′/ 5′-GCGTACTTCTCGCCATCAAACG-3′ and cop-s/ cop-as (see above) for endogenous *copia* promoter region and ORF, respectively; 5′-ACCACGCCCAACCCCCAA-3′/5′-GCTGGTGGAGG TACGGAGACAG-3′ and Het-s2/Het-as2 (see above),

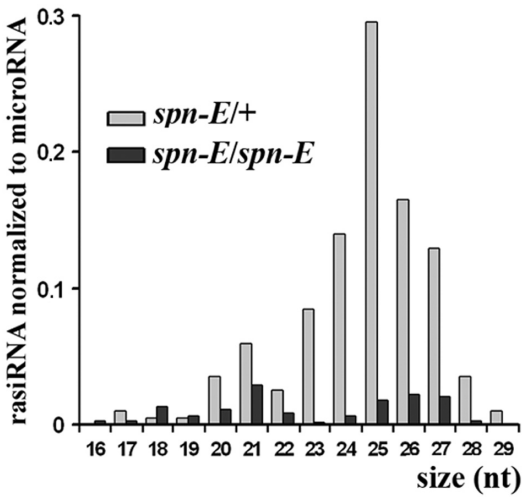

Figure 1. The *spn-E*^{1} mutation leads to the decrease of the overall rasiRNA abundance in ovaries. Length distribution of cloned rasiRNA and the ratios of rasiRNA to microRNA amounts are indicated by bars.

corresponding to *HeT-A* promoter region and ORF, respectively; 5′-CGTGCCTCTCAGTCTAAAGCCTC-3′/5′-CCCGGATTAGCGGTATTGTTGTT-3′ and I el s2/I el as2 (see above), corresponding to *I* element promoter and ORF, respectively; adh d2 and adh r2 (see above), corresponding to *Adh* gene; rp49 s2 and rp49 as2 (see above), corresponding to *rp49* gene; 5′-CGGC GAGGGGGGAAAAGGAC-3′ and 5′-CTTGGCAGC AGGTGGAAAATGTT-3′, corresponding to the 60D intergenic spacer.

RESULTS

The presence of rasiRNAs corresponding to a wide range of transposable elements requires Spn-E function

The *spn-E* (*spindle-E*, *homeless*) gene encodes a putative DExH box RNA helicase, which is required for rasiRNA-mediated silencing of selfish elements (28–30). Previously it was shown that the *spn-E*^{1} mutation leads to the loss of testis short RNAs related to the *Su(Ste)* repeats (2) and ovarian short RNAs of the *HeT-A* LINE element (30) and *roo* LTR retrotransposon (3). To address the effect of the *spn-E* gene on total rasiRNA abundance, we cloned short RNAs from *spn-E*^{1} homo and heterozygous ovaries and testes (Supplementary Table 1). In ovaries the quantity of rasiRNAs was 5-fold higher than that of miRNAs. This is a drastically increased ratio compared to the one calculated previously for *Drosophila* embryos and adult flies (about 0.65 and 0.1, respectively) (1). In contrast to ovaries, approximately equal amounts of microRNAs and rasiRNAs were observed in testes. The amount of rasiRNAs cloned from homozygous *spn-E*^{1} ovaries was 6.7 and 3.3 times lower than in heterozygotes if normalized to microRNA or to the sum of cloned fragments of ribosomal and transfer RNA, respectively (Figure 1). Both sense and antisense rasiRNAs abundance was decreased in *spn-E*^{1} homozygous ovaries. *spn-E*^{1} exerted the most pronounced effects on the amount of rasiRNAs related to LINE elements (*Doc*, *F-element*, *G2* and *R1A1*) and some LTR retrotransposons (*GATE*, *gypsy6* and *MAX-element*). The total amount of LINE-related rasiRNAs normalized to miRNAs was 20-fold lower in homozygous *spn-E*^{1} ovaries, whereas only a 4-fold decrease of LTR retrotransposon rasiRNA abundance was revealed (Supplementary Table 1).

Derepression of transposable elements in the germline correlates with opening of chromatin structure

To investigate the role of chromatin state in rasiRNA-mediated transposable element silencing, we performed ChIP analysis of chromatin in ovarian nuclei using antibodies specific to known histone modifications. We focused on the three extensively investigated retrotransposons of *Drosophila melanogaster*: *I* element, *HeT-A* (LINE elements) and LTR-containing *copia* element. These three retrotransposons were shown to be up-regulated due to *spn-E*^{1} and other mutations, affecting the rasiRNA pathway in flies (3,28,30).

In *spn-E*^{1}/+ heterozygous ovaries the chromatin of promoter and coding regions of tested retrotransposons compared with that of the ORF of the ribosomal *rp49* gene contained a significantly lower level of histone H3 dimethylated at lysine 4 (H3 K4me2), the principal mark of transcriptionally active chromatin (31) (Figure 2). On the contrary, chromatin of retrotransposons was enriched with H3 K9me2 and particularly with H3 K9me3 mark, which are specific for inactive chromatin (32,33) (Figure 2). In *spn-E*^{1} homozygous ovaries we observed an increase in H3 K4me2 and a decrease in H3 K9me3 in promoters, as well as in coding regions of retrotransposons, but not in the chromatin of *rp49* and *Adh* genes (Figure 2). Since methylation of H3 K4 was shown to be a cotranscriptional process (34), the increase in H3 K4me2 in the chromatin of retrotransposon coding regions may be considered as a consequence of an elevated level of their transcription.

Along with endogenous retroelements, we performed ChIP analysis of a transgenic construct containing the reporter *lacZ* gene driven by *copia* LTR (*copia*LTR-*lacZ*) located on the X chromosome. We also observed an increase of H3 K4me2 occupancy in *spn-E*^{1} homozygous ovaries, but no decrease of the repressive H3 K9me2 and H3 K9me3 marks (Supplementary Figure 2). The absence of this latter effect may be attributed to the euchromatic location of the *copia*LTR-*lacZ* transgene compared to the mainly heterochromatic locations of endogenous *copia* elements.

The level of TAF1 protein, which is a known component of RNA polymerase II transcription initiation complex TFIID (35), remained unchanged in *spn-E*^{1} homozygous ovaries in *HeT-A* and *copia* promoters. The TAF1 level was increased 3-fold in the *I* element promoter (Figure 2) and increased 2-fold in *copia*LTR-*lacZ* transgenic construct (Supplementary Figure 2). These results allow us to propose that chromatin opening is unlikely to occur as a result of enrichment with basal transcription factors in promoter regions.

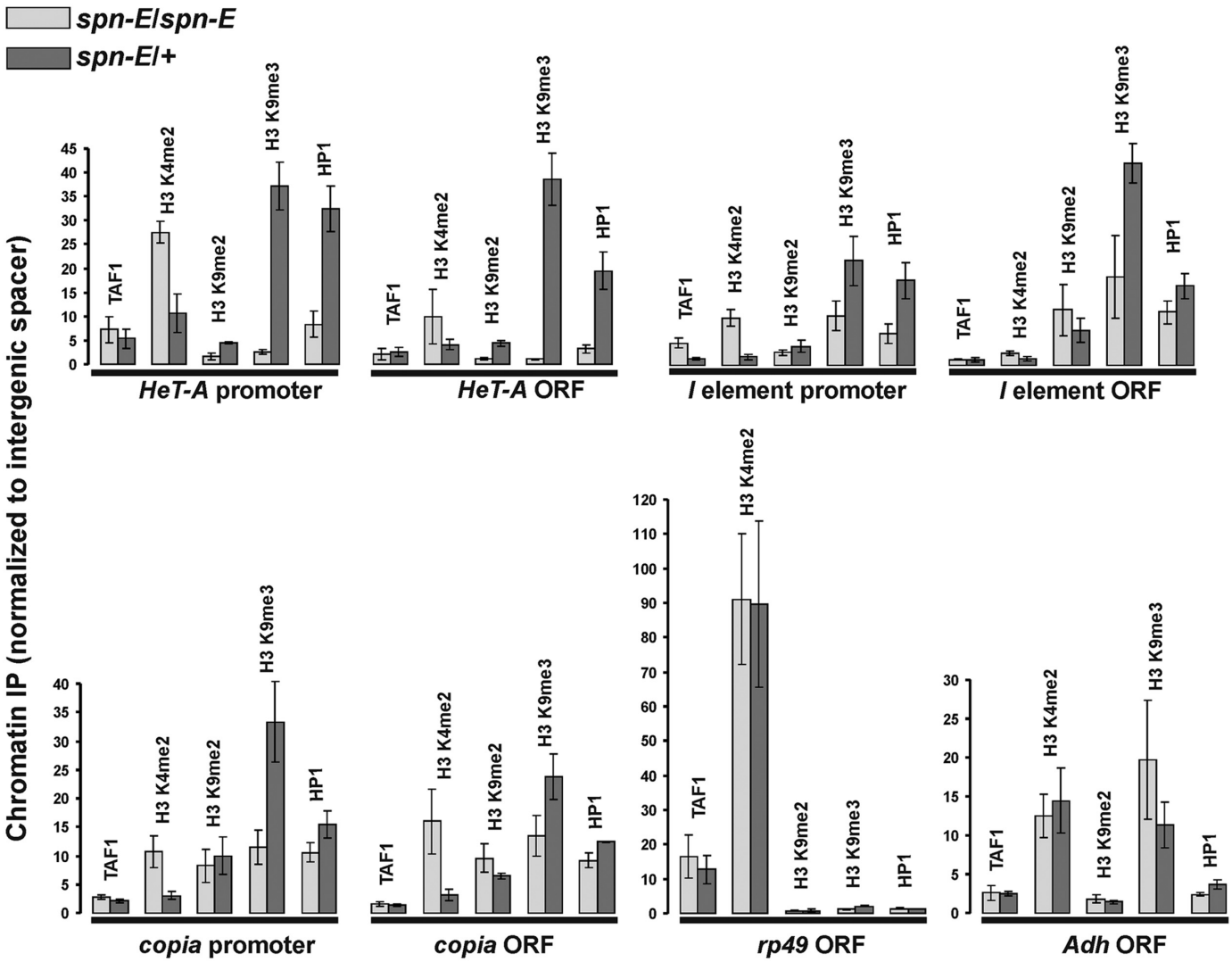

Figure 2. ChIP analysis of retrotransposons in ovarian chromatin. TAF1 occupancy and histone modifications in the chromatin of *spn-E^{1}/spn-E^{1}* (light bars) and *spn-E^{1}/+* ovaries (dark bars) were tested. DNA in precipitates was measured by quantitative real-time PCR using primers to promoter and coding regions (ORF) of LINEs (*HeT-A* and *I* element) and LTR retrotransposon *copia*. The level of H3 K4me2 histone modification typical of transcriptionally active chromatin is significantly lower in retrotransposons, than in ORF of the constitutive rp49 gene. The *spn-E^{1}* mutation increases the level of H3 K4me2 and decreases the level of H3 K9me3 and HP1 both in promoters and ORFs of retrotransposons, but not in chromatin of *rp49* and *Adh* genes. The obtained data were normalized to the fragment of intergenic non-transcribed spacer, located in the 60D region. The amplified spacer fragment contains no binding sites for any known chromatin proteins and does not belong to any repeat that may be silenced.

We detected a significant amount of heterochromatic protein HP1 in the chromatin of *I* element, *HeT-A* and *copia* retrotransposons in ovaries. *spn-E^{1}* caused reduction of HP1 content in retrotransposons, especially for *HeT-A* (Figure 2). The 4-fold decrease of HP1 level was observed in promoters and 6-fold decrease in coding regions of *HeT-A*. At the same time, mutations in the HP1-encoding gene, which are available only in heterozygous state, lead to a drastic accumulation of *HeT-A* transcripts (36,37). This indicates that even a 2-fold decrease of HP1 level is sufficient for *HeT-A* derepression and the observed loss of HP1 occupancy of *HeT-A* chromatin, owing to *spn-E^{1}*, causes transcriptional activation.

The most pronounced changes of the histone marks and HP1 level in *HeT-A* chromatin correlates with the most dramatic increase of *HeT-A* transcript level, compared to *copia* and *I* element in *spn-E^{1}* ovaries (Figure 3).

rasiRNA-mediated chromatin silencing is restricted to the germline

It was demonstrated that SPN-E, PIWI and AUB proteins are required for heterochromatin formation in somatic tissues of *D. melanogaster* (17). Some rasiRNA-pathway components have also been shown to be required for nuclear organization of a chromatin insulator (38), functioning of Polycomb chromatin complexes (39) and

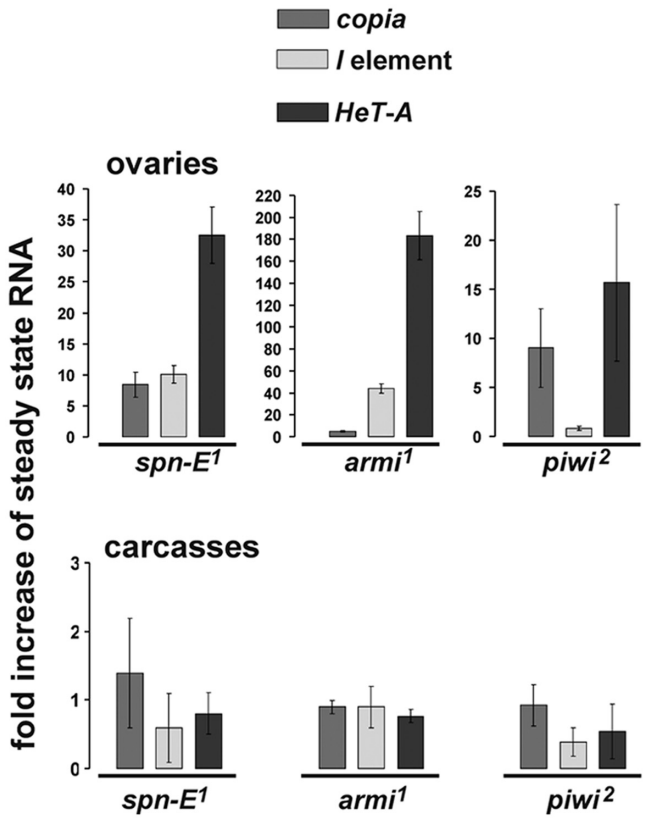

Figure 3. Increase of retrotransposon transcript abundance in homozygous *spn-E*^{1}, *piwi*^{2} and *armi*^{1} ovaries, but not in somatic tissues. The steady-state level of transcripts corresponding to *HeT-A*, *I* element and *copia* retrotransposons and house-keeping genes (*rp49* and *Adh*) was detected by quantitative RT–PCR in ovaries and carcasses (flies without ovaries) of heterozygous or homozygous mutant females. Bars indicate the ratio of transcript amount in homozygous flies to heterozygous ones normalized to *Adh* transcript amount. *piwi*^{2} mutation produces less pronounced effects that may result from the severe morphological ovarian defects induced by this mutation.

variegated repression of a *white* reporter carried by the *1360* element (40) in somatic tissues. The origin of short RNAs in these cases remains unknown. We investigated the regulation of *Het-A*, *I* element and *copia* in somatic tissues of flies, carrying mutations in the *spn-E*, *piwi* and *armi* genes, which control the rasiRNA pathway (3). The steady-state levels of *HeT-A*, *I* element and *copia* related to *rp49* and *Adh* transcripts were comparable in ovaries, heads and carcasses (flies without ovaries) of *spn-E*^{1}/+, *piwi*^{2}/+ and *armi*^{1}/+ heterozygous flies, indicating that tested retrotransposons are not exclusively germ-line transcribed. Nevertheless, we observed up-regulation of retrotransposon transcripts only in ovaries, but not in carcasses or heads of homozygous *spn-E*^{1}, *piwi*^{2} and *armi*^{1} flies (Figure 3;data not shown). Furthermore, we found no effects of the *spn-E*^{1} mutation on the histone modifications in carcasses (Figure 4).

To extend the analysis of retrotransposon expression in somatic tissues, we used a transgenic *copia*LTR-*lacZ* construct (Figure 5A). Activity of β-gal increased 10, 9 and 13 times in extracts of homozygous *spn-E*$^{1/hls3987}$,

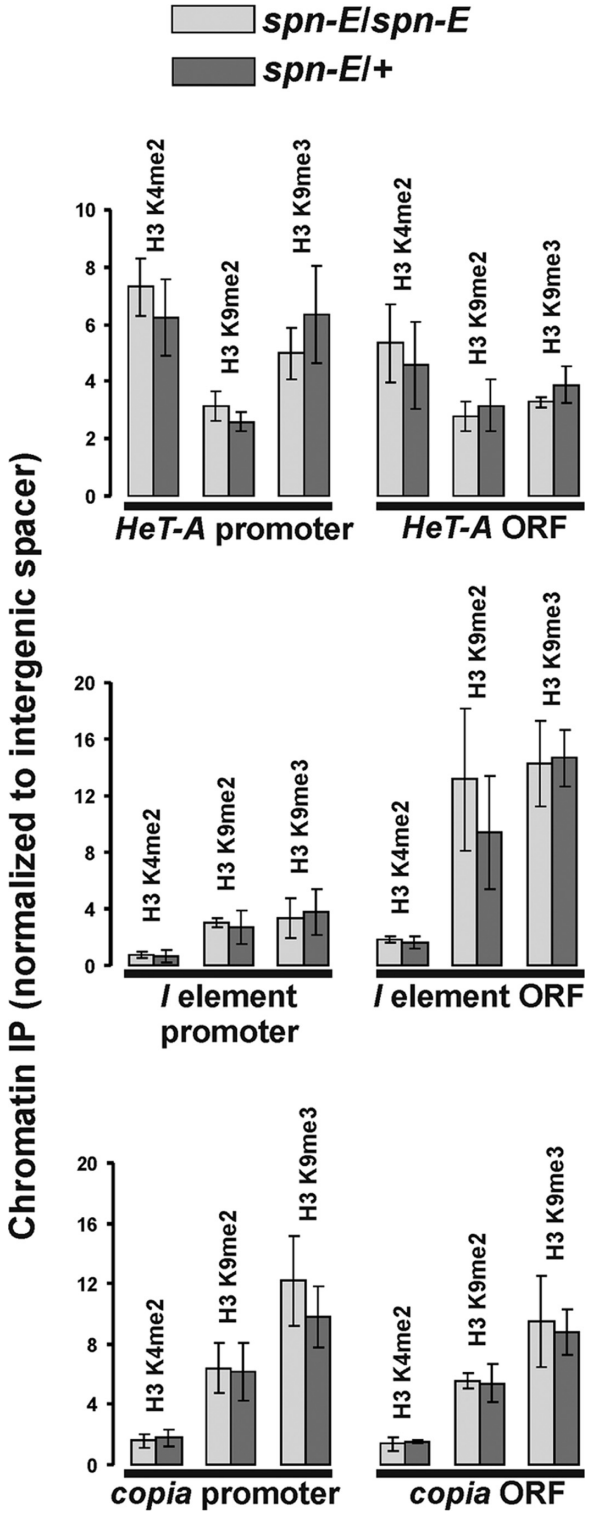

Figure 4. ChIP assay in carcasses. The occupancy of histone modifications in the chromatin of retrotransposons in *spn-E*^{1}/*spn-E*^{1} (light bars) and *spn-E*^{1}/+ ovaries (dark bars).

piwi^{2} and *armi*^{1} ovaries, respectively, as compared to heterozygous ovaries, whereas the expression level remained unchanged in carcasses (Figure 5B). Expression of the construct was dramatically increased in germinal nurse cells and developing oocytes of *spn-E*^{1}, *piwi*^{2}, *armi*^{1}

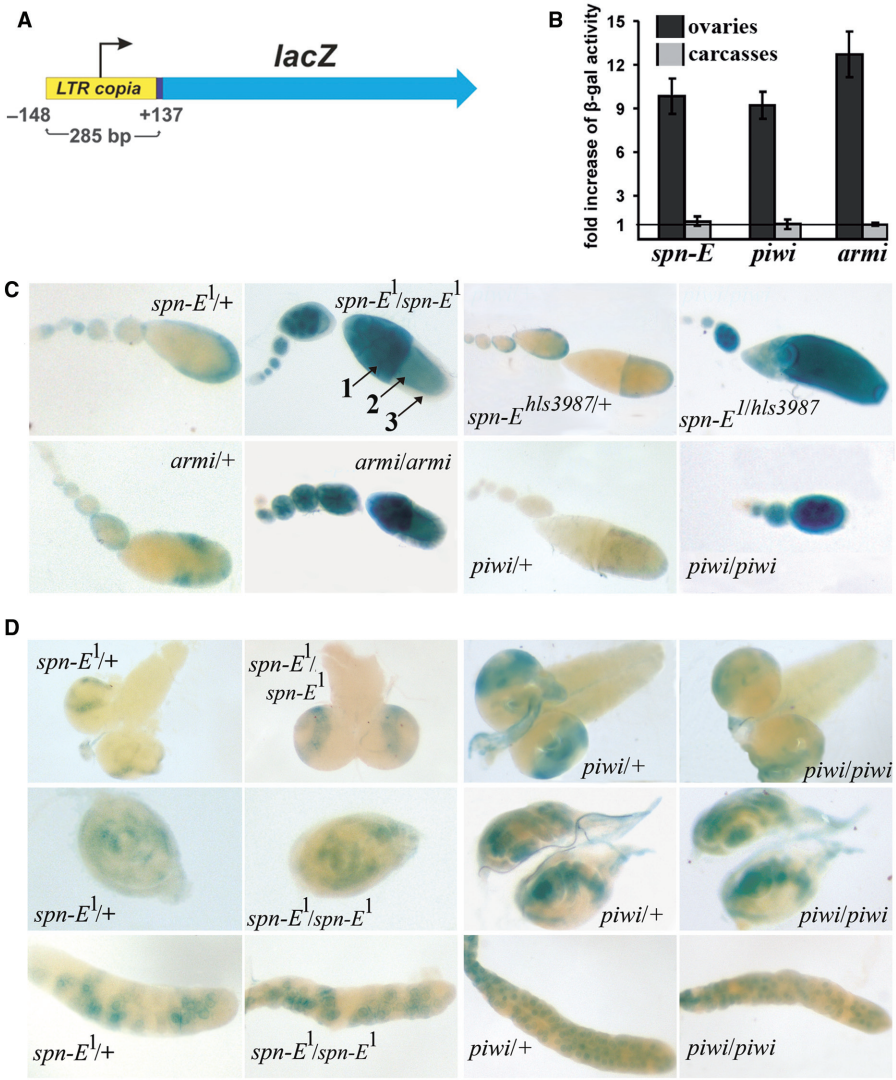

Figure 5. Structure and expression of the reporter construct *copia*LTR-*lacZ*. (**A**) *copia* LTR comprises the known upstream regulatory region, including 137 bp of the transcribed fragment. (**B**) β-gal activity in ovaries and carcasses. Bars indicate the ratio of β-gal activity in *spn-E*$^{1/hls3987}$ trans-heterozygous and *piwi*^{2}, *armi*^{1} homozygous to heterozygous flies. The strong increase in β-gal activity due to the mutations was revealed in ovarian extracts (dark bars), whereas no significant changes were detected in extracts of carcasses (gray bars). Measurements of β-gal activity were normalized to total level of protein in extracts. (**C**) Expression of *copia*LTR-*lacZ* located on the X-chromosome in *spn-E*^{1}, *piwi*^{2}, *armi*^{1} homozygous and trans-heterozygous *spn-E*$^{1/hls3987}$ ovaries. In heterozygous *spn-E*^{1}/+, *spn-E*^{hls3987}/+, *piwi*^{2}/+ and *armi*^{1}/+ ovaries *lacZ* expression occurs at a low level. In ovaries of homozygous and trans-heterozygous (*spnE*$^{1/hls3987}$) females expression is increased in germinal nurse cells (arrow 2) and developing oocyte (arrow 1), but not in somatic follicle cells (arrow 3). (**D**) *lacZ* expression level is not increased in somatic organs of *spn-E*^{1} and *piwi*^{2} larvae. *lacZ* staining of larval brain (first row), haltere imaginal discs (second row) and salivary glands (third row) is shown.

homozygous and *spn-E*$^{1/hls3987}$ trans-heterozygous ovaries (Figure 5C). *lacZ* expression level remained unchanged in brain, imaginal discs and salivary glands of *spn-E*^{1}, *piwi*^{2} and *armi*^{1} larvae as compared with heterozygous or wild-type controls (Figure 5D; data not shown). Thus, the chromatin-based regulation of tested retrotransposons mediated by rasiRNAs is realized in the germline.

DISCUSSION

We demonstrated that short rasiRNA species, known to be associated with Piwi subfamily proteins (3–6), have a germline-specific function in the maintenance of chromatin modifications of retrotransposons. The s*pn-E*, *piwi* and *armi* genes are predominantly expressed in germ cells and their mutant states lead to abnormalities in germ-line development and sterility (41–44). Moreover, evidence of germ-line specificity of the rasiRNA-mediated silencing pathway is supported by the observation, that rasiRNAs are significantly more abundant in the germline than in somatic tissues. Germline-specific silencing of mobile elements is considered an important defense mechanism against mutations caused by mobile element transpositions, because selfish transposable elements are thought to be expressed mainly in germinal cells to ensure their amplification and transmission to the progeny. A distinct function of rasiRNA-mediated silencing concerns the maintenance of *Drosophila* telomeric state. Extension

of telomeres is realized by germ-line specific transpositions of *HeT-A, TAHRE* and *TART* LINE elements (45–47). The *aub* and *spn-E* genes are implicated in the control of *HeT-A* and *TART* expression, accumulation of corresponding rasiRNAs and frequency of *HeT-A* and *TART* attachment to broken chromosome ends in ovaries (30). Thus, here we demonstrated the involvement of the rasiRNA pathway in the chromatin modification of beneficial telomeric retrotransposons and dangerous transposable elements.

We found that elimination of rasiRNAs in *Drosophila* ovaries caused by *spn-E*^{1} leads to the decompaction of chromatin of retrotransposons. The decrease of HP1 level and the changes in histone modification patterns, manifesting itself in an increase of H3 K4me2 and decrease of H3 K9me3 were observed. A correlation between the most dramatic increase of *HeT-A* transcript abundance, owing to the *spn-E*^{1}, *piwi*^{2} and *armi*^{1} mutations (Figure 3), and the most significant changes of chromatin structure caused by *spn-E*^{1} compared to the *I* element, *copia* and *copia*LTR-*lacZ* (Figure 2, Supplementary Figure 2) suggest that changes of chromatin structure in ovaries of rasiRNA mutants are accompanied by transcriptional activation of retrotransposons. At the same time, we detected no effects of *spn-E*^{1} on the chromatin state of retrotransposons in somatic tissues. The observed germline specificity of rasiRNA-mediated retrotransposon silencing is in apparent contradiction with the observations that the *spn-E*, *piwi* and *aub* mutations affect heterochromatin formation in somatic tissues (17,48) and these genes are required for variegated repression of a *white* reporter carried by the *1360* element (40). It is appropriate to point out that the size of the short RNAs corresponding to the *1360* element (40) and transgenic *Fab-7* copies (39) (~23 nt) in somatic tissues is consistent with Dicer-produced siRNAs, but not with rasiRNAs, suggesting that silencing of mobile elements in somatic tissues may be realized via RNAi, but not the rasiRNA pathway. Alternatively, rasiRNA-dependent heterochromatin formation might be induced in early stages of embryonic development and then be epigenetically inherited in somatic tissues in a rasiRNA-independent manner.

The mechanism of chromatin modification caused by rasiRNAs remains obscure. Although the Piwi protein was shown to be localized in cell nuclei (4,6,42), we failed to detect Piwi in the chromatin of retrotransposons (data not shown). Possibly, Piwi is associated with the nascent RNA but may easily leave chromatin. It has been suggested that rasiRNAs direct cleavage of retrotransposon transcripts (4–6). We propose that slicing of the nascent transcript mediated by the Piwi protein is capable to transform RNA polymerase II to a silencing complex. A similar model has been put forward to explain the spreading of transcriptional silencing in fission yeast *Schizosaccharomyces pombe*. It has been suggested that the sliced nascent transcript might recruit the silencing machinery to perform chromatin modification (49,50). Further experiments are required to verify this model of chromatin silencing in the *D. melanogaster* genome.

Our observations emphasize the proposed role of rasi(pi)RNAs in the formation of heterochromatin enriched by mobile elements and other repeats. Heterochromatin serves as a genome region to recruit and spread regulatory proteins to control chromosomal processes, including transcription as well as chromosome segregation. Actually, the disturbance of silencing of the *Stellate* repeats in the *D. melanogaster* genome is accompanied by chromosome meiotic non-disjunctions (51–53) and was shown to be triggered by *spn-E* mutations (22,51). Interestingly, spn-E mutations also lead to breakages in ovarian chromosomes (44) that might be caused by chromatin opening. The peculiarities of rasiRNA-dependent chromatin modification in *Drosophila* male and female germinal cells require further detailed studies taking into account the known role of heterochromatin in chromosome mechanics (54).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Alla Kalmykova for technical support in isolation of ovarian RNA used for short RNA cloning, Olesya Sokolova and Alina Korbut for help in RT-PCR and ChIP experiments, E.G. Pasyukova for kindly providing flies carrying the *copia*LTR-*lacZ* construct and James M. Mason for help in the manuscript preparation. This work was supported by RAS program for Molecular and Cell Biology, Russian Foundation for Basic Research (05-04-48034), the program of Scientific School support (6113.2006.4) and grant of President of Russian Federation for young scientists (02.120.11.9326). Funding to pay the Open Access publication charges for this article was provided by Russian Foundation for Basic Research (05-04-48034).

Conflict of interest statement. None declared.

REFERENCES

1. Aravin,A.A., Lagos-Quintana,M., Yalcin,A., Zavolan,M., Marks,D., Snyder,B., Gaasterland,T., Meyer,J. and Tuschl,T. (2003) The Small RNA profile during Drosophila melanogaster development. *Dev. Cell*, **5**, 337–350.
2. Aravin,A.A., Klenov,M.S., Vagin,V.V., Bantignies,F., Cavalli,G. and Gvozdev,V.A. (2004) Dissection of a natural RNA silencing process in the Drosophila melanogaster germ line. *Mol. Cell Biol.*, **24**, 6742–6750.
3. Vagin,V.V., Sigova,A., Li,C., Seitz,H., Gvozdev,V. and Zamore,P.D. (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. *Science*, **313**, 320–324.
4. Saito,K., Nishida,K.M., Mori,T., Kawamura,Y., Miyoshi,K., Nagami,T., Siomi,H. and Siomi,M.C. (2006) Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. *Genes Dev.*, **20**, 2214–2222.
5. Gunawardane,L.S., Saito,K., Nishida,K.M., Miyoshi,K., Kawamura,Y., Nagami,T., Siomi,H. and Siomi,M.C. (2007) A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in Drosophila. *Science*, **315**, 1587–1590.
6. Brennecke,J., Aravin,A.A., Stark,A., Dus,M., Kellis,M., Sachidanandam,R. and Hannon,G.J. (2007) Discrete small

RNA-generating loci as master regulators of transposon activity in Drosophila. *Cell*, **128**, 1089–1103.
7. Aravin,A.A., Sachidanandam,R., Girard,A., Fejes-Toth,K. and Hannon,G.J. (2007) Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science*, **316**, 744–747.
8. Carmell,M.A., Girard,A., van de Kant,H.J., Bourc'his,D., Bestor,T.H., de Rooij,D.G. and Hannon,G.J. (2007) MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Dev. Cell*, **12**, 503–514.
9. Houwing,S., Kamminga,L.M., Berezikov,E., Cronembold,D., Girard,A., van den Elst,H., Filippov,D.V., Blaser,H., Raz,E. *et al.* (2007) A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. *Cell*, **129**, 69–82.
10. Aravin,A., Gaidatzis,D., Pfeffer,S., Lagos-Quintana,M., Landgraf,P., Iovino,N., Morris,P., Brownstein,M.J., Kuramochi-Miyagawa,S. *et al.* (2006) A novel class of small RNAs bind to MILI protein in mouse testes. *Nature*, **442**, 203–207.
11. Girard,A., Sachidanandam,R., Hannon,G.J. and Carmell,M.A. (2006) A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature*, **442**, 199–202.
12. Lau,N.C., Seto,A.G., Kim,J., Kuramochi-Miyagawa,S., Nakano,T., Bartel,D.P. and Kingston,R.E. (2006) Characterization of the piRNA complex from rat testes. *Science*, **313**, 363–367.
13. Okamura,K., Ishizuka,A., Siomi,H. and Siomi,M.C. (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev.*, **18**, 1655–1666.
14. Lippman,Z. and Martienssen,R. (2004) The role of RNA interference in heterochromatic silencing. *Nature*, **431**, 364–370.
15. Matzke,M.A. and Birchler,J.A. (2005) RNAi-mediated pathways in the nucleus. *Nat. Rev. Genet.*, **6**, 24–35.
16. Wassenegger,M. (2005) The role of the RNAi machinery in heterochromatin formation. *Cell*, **122**, 13–16.
17. Pal-Bhadra,M., Leibovitch,B.A., Gandhi,S.G., Rao,M., Bhadra,U., Birchler,J.A. and Elgin,S.C. (2004) Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery. *Science*, **303**, 669–672.
18. Gillespie,D.E. and Berg,C.A. (1995) Homeless is required for RNA localization in Drosophila oogenesis and encodes a new member of the DE-H family of RNA-dependent ATPases. *Genes Dev.*, **9**, 2495–2508.
19. Gonzalez-Reyes,A., Elliott,H. and St Johnston,D. (1997) Oocyte determination and the origin of polarity in Drosophila: the role of the spindle genes. *Development*, **124**, 4927–4937.
20. Lin,H. and Spradling,A.C. (1997) A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. *Development*, **124**, 2463–2476.
21. Cook,H.A., Koppetsch,B.S., Wu,J. and Theurkauf,W.E. (2004) The Drosophila SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification. *Cell*, **116**, 817–829.
22. Aravin,A.A., Naumova,N.M., Tulin,A.V., Vagin,V.V., Rozovsky,Y.M. and Gvozdev,V.A. (2001) Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline. *Curr. Biol.*, **11**, 1017–1027.
23. Lagos-Quintana,M., Rauhut,R., Yalcin,A., Meyer,J., Lendeckel,W. and Tuschl,T. (2002) Identification of tissue-specific microRNAs from mouse. *Curr. Biol.*, **12**, 735–739.
24. Pfeffer,S., Lagos-Quintana,M. and Tuschl,T. (2003) *Cloning of Small RNA Molecules* Wiley and Sons, New York, NY.
25. Altschul,S.F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**, 3389–3402.
26. Stajich,J.E., Block,D., Boulez,K., Brenner,S.E., Chervitz,S.A., Dagdigian,C., Fuellen,G., Gilbert,J.G., Korf,I. *et al.* (2002) The Bioperl toolkit: Perl modules for the life sciences. *Genome Res.*, **12**, 1611–1618.
27. Chanas,G., Lavrov,S., Iral,F., Cavalli,G. and Maschat,F. (2004) Engrailed and polyhomeotic maintain posterior cell identity through cubitus-interruptus regulation. *Dev. Biol.*, **272**, 522–535.
28. Vagin,V.V., Klenov,M.S., Kalmykova,A.I., Stolyarenko,A.D., Kotelnikov,R.N. and Gvozdev,V.A. (2004) The RNA interference proteins and vasa locus are involved in the silencing of retrotransposons in the female germline of Drosophila melanogaster. *RNA Biol.*, **1**, 54–58.
29. Kalmykova,A.I., Klenov,M.S. and Gvozdev,V.A. (2005) Argonaute protein PIWI controls mobilization of retrotransposons in the Drosophila male germline. *Nucleic Acids Res.*, **33**, 2052–2059.
30. Savitsky,M., Kwon,D., Georgiev,P., Kalmykova,A. and Gvozdev,V. (2006) Telomere elongation is under the control of the RNAi-based mechanism in the Drosophila germline. *Genes Dev.*, **20**, 345–354.
31. Schubeler,D., MacAlpine,D.M., Scalzo,D., Wirbelauer,C., Kooperberg,C., van Leeuwen,F., Gottschling,D.E., O'Neill,L.P., Turner,B.M. *et al.* (2004) The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. *Genes Dev.*, **18**, 1263–1271.
32. Martin,C. and Zhang,Y. (2005) The diverse functions of histone lysine methylation. *Nat. Rev. Mol. Cell Biol.*, **6**, 838–849.
33. Ebert,A., Lein,S., Schotta,G. and Reuter,G. (2006) Histone modification and the control of heterochromatic gene silencing in Drosophila. *Chromosome Res.*, **14**, 377–392.
34. Eissenberg,J.C. and Shilatifard,A. (2006) Leaving a mark: the many footprints of the elongating RNA polymerase II. *Curr. Opin. Genet. Dev.*, **16**, 184–190.
35. Thomas,M.C. and Chiang,C.M. (2006) The general transcription machinery and general cofactors. *Crit. Rev. Biochem. Mol. Biol.*, **41**, 105–178.
36. Savitsky,M., Kravchuk,O., Melnikova,L. and Georgiev,P. (2002) Heterochromatin protein 1 is involved in control of telomere elongation in Drosophila melanogaster. *Mol. Cell Biol.*, **22**, 3204–3218.
37. Perrini,B., Piacentini,L., Fanti,L., Altieri,F., Chichiarelli,S., Berloco,M., Turano,C., Ferraro,A. and Pimpinelli,S. (2004) HP1 controls telomere capping, telomere elongation, and telomere silencing by two different mechanisms in Drosophila. *Mol. Cell*, **15**, 467–476.
38. Lei,E.P. and Corces,V.G. (2006) RNA interference machinery influences the nuclear organization of a chromatin insulator. *Nat. Genet.*, **38**, 936–941.
39. Grimaud,C., Bantignies,F., Pal-Bhadra,M., Ghana,P., Bhadra,U. and Cavalli,G. (2006) RNAi components are required for nuclear clustering of Polycomb group response elements. *Cell*, **124**, 957–971.
40. Haynes,K.A., Caudy,A.A., Collins,L. and Elgin,S.C. (2006) Element 1360 and RNAi components contribute to HP1-dependent silencing of a pericentric reporter. *Curr. Biol.*, **16**, 2222–2227.
41. Cox,D.N., Chao,A., Baker,J., Chang,L., Qiao,D. and Lin,H. (1998) A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes Dev.*, **12**, 3715–3727.
42. Cox,D.N., Chao,A. and Lin,H. (2000) Piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development*, **127**, 503–514.
43. Williams,R.W. and Rubin,G.M. (2002)) ARGONAUTE1 is required for efficient RNA interference in Drosophila embryos. *Proc. Natl Acad. Sci. USA*, **99**, 6889–6894.
44. Klattenhoff,C., Bratu,D.P., McGinnis-Schultz,N., Koppetsch,B.S., Cook,H.A. and Theurkauf,W.E. (2007) Drosophila rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. *Dev. Cell*, **12**, 45–55.
45. Biessmann,H. and Mason,J.M. (2003) Telomerase-independent mechanisms of telomere elongation. *Cell Mol. Life Sci.*, **60**, 2325–2333.
46. Melnikova,L. and Georgiev,P. (2005) Drosophila telomeres: the non-telomerase alternative. *Chromosome Res.*, **13**, 431–441.
47. Casacuberta,E. and Pardue,M.L. (2005) HeT-A and TART, two Drosophila retrotransposons with a bona fide role in chromosome structure for more than 60 million years. *Cytogenet. Genome Res.*, **110**, 152–159.

48. Kavi,H.H., Fernandez,H.R., Xie,W. and Birchler,J.A. (2005) RNA silencing in Drosophila. *FEBS Lett*., **579**, 5940–5949.
49. Irvine,D.V., Zaratiegui,M., Tolia,N.H., Goto,D.B., Chitwood,D.H., Vaughn,M.W., Joshua-Tor,L. and Martienssen,R.A. (2006) Argonaute slicing is required for heterochromatic silencing and spreading. *Science*, **313**, 1134–1137.
50. Zaratiegui,M., Irvine,D.V. and Martienssen,R.A. (2007) Noncoding RNAs and gene silencing. *Cell*, **128**, 763–776.
51. Stapleton,W., Das,S. and McKee,B.D. (2001) A role of the Drosophila homeless gene in repression of Stellate in male meiosis. *Chromosoma*, **110**, 228–240.
52. Belloni,M., Tritto,P., Bozzetti,M.P., Palumbo,G. and Robbins,L.G. (2002) Does Stellate cause meiotic drive in Drosophila melanogaster? *Genetics*, **161**, 1551–1559.
53. Boschi,M., Belloni,M. and Robbins,L.G. (2006) Genetic evidence that nonhomologous disjunction and meiotic drive are properties of wild-type Drosophila melanogaster male meiosis. *Genetics*, **172**, 305–316.
54. Dernburg,A.F., Sedat,J.W. and Hawley,R.S. (1996) Direct evidence of a role for heterochromatin in meiotic chromosome segregation. *Cell*, **86**, 135–146.